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Oral Toxicity to Flesh Flies of a Neurotoxic Polypeptide

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An insect selective neurotoxic polypeptide from venom of the scorpion *Androctonus australis* (AaIT, M_r 8,000) was shown to cross the midgut of the flesh fly *Sarcophaga falculata*, using assays of oral toxicity, column chromatography, and microscopic autoradiography of the native and radioiodinated toxin. AaIT induced paralysis of flies within 1–2 h after oral administration, with a lethal dose (LD_{50}) of 10 μg/100 mg of body weight. Oral toxicity was about 0.14% of toxicity by injection. Hemolymph collection 70–85 min after feeding flies with [^{125}I]AaIT showed that 5% of ingested radioactivity appeared in hemolymph. Most of this represented degradation products, but included about 0.3% of the chromatographically intact toxin. In contrast, hemolymph of identically treated lepidopterous larvae (*Manduca, Helioverpa* [=Heliothis]) contained degradation products but no intact toxin. [^{125}I]AaIT was shown to cross the midgut of *Sarcophaga* through a morphologically distinct segment of the midgut previously shown to be permeable to a cytotoxic, positively charged polypeptide of similar molecular weight. These results suggest that *Sarcophaga* midgut contains a morphologically and functionally distinct segment that transports small peptides, and that employment of neurotoxic polypeptides for insect control may be feasible. Activity might be greatly improved through modification and metabolic stabilization of active peptides.

Key words: polypeptide neurotoxin, gut permeability, *Sarcophaga falculata*

INTRODUCTION

Previous work showed that a cobra venom cardiotoxin, an amphipathic and positively charged polypeptide of 7,000 molecular weight, was orally toxic to *Sarcophaga* flesh flies [1,2]. A crucial factor in cardiotoxin toxicity was its ability to cross the digestive system into the hemocoel [2] through a morphologically

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distinct segment of the midgut [3]. Penetration of the cardiotoxin through midgut membranes was attributed to its ability to interact with biological membranes through association with their phospholipid components [4,5], and perhaps with phospholipids specific to the cardiotoxin-permeable segment of the midgut [3].

We therefore tested whether a polypeptide with chemical and pharmacological properties differing from those of cardiotoxin is also able to penetrate the digestive system of the flesh fly. AaIT,* an insect-selective neurotoxin isolated from the venom of the scorpion *Androctonus australis,* has recently been shown to affect house flies when topically applied [6]. In contrast to cardiotoxin, which interacts with many biological membranes, AaIT is a hydrophilic polypeptide (M_r 8,000) which possesses only a single class of binding sites located exclusively on insect neuronal membranes [7–10].

MATERIALS AND METHODS

Biochemicals

The crude venom of the scorpion *A. australis* was purchased from Latoxan (Rosan, France). AaIT was purified from the venom by column chromatography according to a previously described method [11].

Inulin[^{3}H]carboxylic acid (2.1 μCi/mg), for the determination of hemolymph volume, and Na[^{125}I], for protein radioiodination, were purchased from Amersham (Amersham, England).

[^{125}I]AaIT with specific activities of 60–80 μCi/μg (480–640 Ci/mmol) was prepared according to Herrmann et al. [12].

Toxicity Assays

Flesh flies of the species *Sarcophaga falculata* (=*argyrostoma*) were bred in the laboratory. Female flies of an average weight of 52.5 ± 6.9 mg (± S.D., n = 10) were deprived of food and water and employed in experiments 24–48 h after eclosion. In oral toxicity assays, the test substance, dissolved in a solution of 1% sucrose in water, was introduced to the proboscis through a calibrated 10 μl syringe (Hamilton, Reno, Nevada, USA) in volumes of 5–10 μl per fly. Ingestion by the fly was improved when a crystal of sucrose was placed on the tip of the needle contacting the proboscis during drinking. Ingestion of the test substance was verified by feeding flies toxin dissolved in a 0.02% solution of the dye erythrosine and observing the movement of ingested solution through surgically exposed digestive tract. In injection assays, flies were injected through the scutum into the thorax with test substances dissolved in 0.65% (w/v) NaCl in water in volumes of 1–4 μl per fly. In both injection and feeding assays (see Table 1), five to seven animals were employed for each dose, and sampling and estimation of the 50% end points, LD_{50} and PD_{50}, were performed according to Reed and Muench [13].

*Abbreviations used: AaIT = *Androctonus australis* insect toxin; BSA = bovine serum albumin; DDT = dichlorodiphenyltrichloroethane; LD_{50} = dose lethal to 50% of a population; PD_{50} = dose paralytic to 50% of a population.

Determination of Hemolymph Volume

Hemolymph volumes were determined according to the method of Wharton et al. [14] by injecting a radiotracer, inulin[^{3}H]carboxylic acid [12], into the thorax and collecting hemolymph through a cut leg.

Column Chromatography

Gel filtration chromatography was performed with Ultrogel AcA 202 (IBF Reactif, Villeneuve-la-Garenne, France), a polyacrylamide-agarose gel of 1,000–15,000 M_r inclusion limits. The gel was washed and equilibrated with the running buffer (0.2 M sodium phosphate, pH 7, 0.04% sodium azide, 0.5 mg/ml BSA), packed in a column, and employed as specified in the legend to Figure 2.

Histological Autoradiography

Female *Sarcophaga* flies were fed 3 μCi of [^{125}I]AaIT in a volume of 7.5 μl. Light microscope autoradiography of the gut was performed at various time intervals after feeding (see Fig. 3). Dissections, fixations, postfixation, dehydration, and embedding were performed according to Fishman et al. [3]. Sections 2–3 μm thick were prepared, treated with Nuclear track emulsion NTB2 (Kodak, Rochester, NY) according to Gude [15], and stored for 7–9 days. The emulsion was developed with Kodak D-19 developer, unstained sections were examined with a phase contrast microscope (Zeiss, Oberkochen, Germany), and double exposures were made, focusing on grains and tissue separately through a 25-power objective.

RESULTS

Toxicity of Crude *A. australis* Venom and AaIT to *Sarcophaga* flies

Table 1 summarizes toxicities of crude venom and AaIT by injection and ingestion. In the injection assays, toxicity was followed according to two criteria: rapid paralysis represented by PD_{50} determined 30 min after the injection, and LD_{50} determined 20–24 h after injection. In the oral treatments, only the LD_{50} was quantitatively estimated but symptoms of paralysis (inability to walk and stand accompanied by occasional bursts of wing movement) were observed within 1–2 h after treatment.

TABLE 1. Toxicities to *Sarcophaga* Flies of Injected and Ingested Crude Venom and AaIT

	Injection		Oral treatment	
	PD_{50}	LD_{50}	LD_{50}	
	(ng/100 mg b.w.[a])		(ng/100 mg b.w.)	Ratio[b]
Crude venom	165	352	275,000	781
AaIT	1.5	14	10,000	718
Ratio of crude venom to AaIT	110	25	27.5	----

[a]b.w. = body weight.
[b]Ratio of oral treatment to injection.

Hemolymph Volume Determination

Determination of hemolymph volume was essential to estimate the total [^{125}I]AaIT penetrating into the hemocoel (see below). Hemolymph volume, determined by injection of radioactive inulin into unfed flies, was 11.5 ± 1 μl/50 mg body weight (± S.D., n = 7). When hemolymph volume was determined by injecting [^{3}H]inulin into flies 1 h after ingestion of 10 μl of 1% sucrose, the average hemolymph volume was 13.3 ± 1.2 μl/50 mg body weight (± S.D., n = 6).

Appearance of Radioactive Toxin in Hemolymph of *Sarcophaga*

To determine whether intact AaIT can penetrate through the midgut into the hemocoel, flies were fed [^{125}I]AaIT in 10 μl of 1% sucrose, and 1–3 μl of hemolymph was collected from a cut leg or from the scutum 70–85 min after feeding. Radioactivity was normalized to the 13.3 μl estimated hemolymph volume and compared to the total radioactivity introduced into the fly (Table 2). The results indicate that an average of 5 ± 1.8% (± S.D., n = 12) of the radioactivity from ingested [^{125}I]AaIT was detected in the hemolymph. For comparison, an average of only 0.4 ± 0.1% (± S.D., n = 5) of activity from ingested radiolabeled inulin (a carbohydrate with an estimated M_r of 5,600) was recovered in the hemolymph.

TABLE 2. Amount of Radioactivity in Hemolymph of *Sarcophaga* Flies 70– 85 Min After Feeding [^{125}I]AaIT

Fly no.	Total ingested radioactivity[a] (cpm × 10^{-3})	Volume sampled (μl)	Radioactivity of sample[b] (cpm × 10^{-3})	Estimated total hemolymph radioactivity[c] (cpm × 10^{-3})	Hemolymph radioactivity (%)
1	41.3	0.5	0.069	1.86	4.5
2	60.2	1.2	0.401	4.44	7.4
3	83.7	2.5	1.101	5.85	7.0
4	120.0	1.5	0.795	7.05	5.9
5	130.1	0.8	0.532	8.85	6.8
6	136.6	0.5	0.198	5.27	3.8
7	148.8	2.0	1.500	10.00	6.7
8	182.8	2.1	1.484	9.40	5.1
9	233.7	1.0	0.414	5.50	2.4
10[d]	461.3	3.0	4.364	19.35	4.2
11[d]	570.6	3.0	4.240	18.80	3.3
12[d]	570.0	3.0	2.776	12.31	2.2

[a]Thirty-five flies were each fed 10 μl of solution with various concentrations of [^{125}I]AaIT, placed in separate vials, and total radioactivity (vial and fly) was measured (data not shown). Seventy to 85 min later, each fly was removed from its vial, placed in a new vial, and the radioactivity of the intact fly was counted. The empty vials from which flies had originally been removed were counted in parallel. The various flies revealed a wide range of 0.5–25% drop in the radioactivity count between the first and the second radioactivity counts of the intact flies. The reduction was due to regurgitation and, to a lesser degree, to excretion as indicated by the radioactivity counted in the empty vials (data not shown). Samplings of hemolymph, for the estimation of gut crossing, were performed only from those flies that revealed a difference lower than 2% between the first and the second counts of the intact flies. These data are presented in the Table.
[b]Corrected for background (115 cpm).
[c]Normalized to 13.3 μl, the average hemolymph volume after feeding 10 μl of solution.
[d]The hemolymph samples were collected from scutum and used for the paper chromatography presented in Figure 1.

To determine whether radioactivity from hemolymph represented the peptide or free radioiodide, samples 10–12 (Table 2) of the radioactive hemolymph were run on ascending paper chromatography in methanol. This procedure is routinely employed in the radioiodination of AaIT to estimate the amount of free iodide vs. yield of iodinated AaIT. Proteins and polypeptides remain at the origin while free iodide is carried behind the front [16]. The results presented in Figure 1 reveal a distribution of radioactivity similar to that of iodinated [^{125}I]AaIT. This indicates that the radioactivity detected in the hemolymph is not due to radioiodide. This result, however, does not exclude the possibility that we were detecting radioiodinated degradation products from the peptide.

About 70,000 cpm of radioactive hemolymph were collected from 15 flies within 60–90 min after ingesting [^{125}I]AaIT, and analyzed on a gel filtration column as specified in Materials and Methods and the legend to Figure 2. As shown in Figure 2, the radioactive *Sarcophaga* hemolymph yielded three radioactive fractions with peaks at elution volumes of 8, 15.5, and 20 ml. From a parallel separation of the [^{125}I]AaIT (Fig. 2), it may be concluded that the

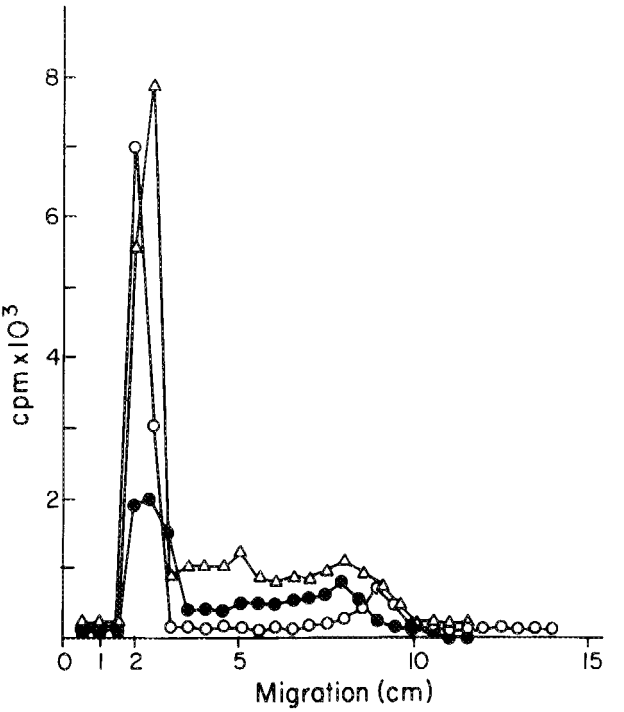

Fig. 1. Paper chromatography of hemolymph collected from *Sarcophaga* flies and *Manduca* larvae fed with [^{125}I]AaIT. Each of the below three samples was applied to a strip (2 × 18 cm) of Whatman No. 1 filter paper (2 cm from the bottom) and chromatographed with methanol for about 30 min. Chromatography was stopped when the eluant reached the level of 15 cm. After drying, the paper strip was cut into segments of 0.5 cm each and the radioactivity of the segments was counted by scintillation. ●, *Sarcophaga* hemolymph collected from flies 10–12 in Table 1, about 1 × 10^4 cpm. △, A single *Manduca* larva (140 mg live weight) was fed with 2.5 × 10^5 cpm of radioactivity and was collected and run on a separate paper strip. ○, [^{125}I]AaIT—2 × 10^4 cpm.

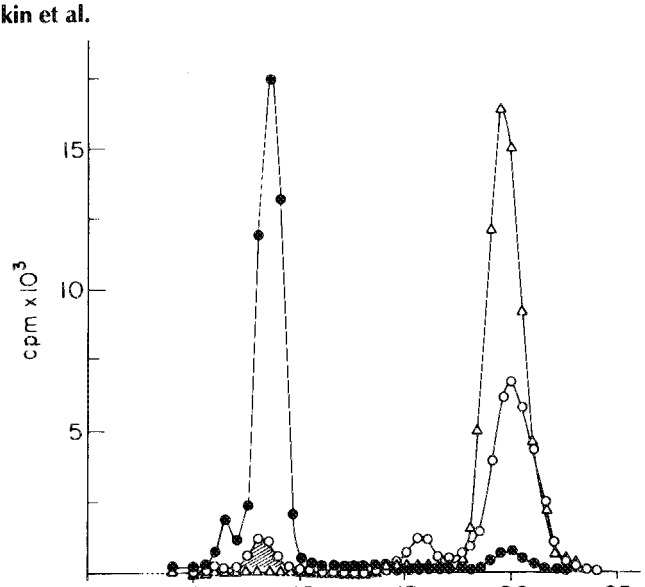

Fig. 2. Gel filtration column chromatography of hemolymph from *Sarcophaga* flies and *Manduca* larvae fed with [^{125}I]AaIT. The column (28 × 0.9 cm) filled with Ultrogel AcA 202 was equilibrated and eluted with running buffer (0.2 M sodium phosphate, pH 7, 0.04% sodium azide; 0.5 mg/ml BSA) at a flowrate of 1.8 ml/h. Fractions of 0.5 ml were collected and their radioactivity was measured by liquid scintillation counting. ○, Radioactive hemolymph (72 μl, 7 × 10^4 cpm) was collected from 15 flies 60–90 min after feeding with the radioactive toxin. The marked fraction corresponds to 6.5% of the total radioactivity eluted from the column. △, Sixty-five microliters of hemolymph with a total radioactivity of 1.1 × 10^5 cpm was collected from three *Manduca* larvae 70 min after being fed 2.5 × 10^5 cpm of [^{125}I]AaIT; ●, [^{125}I]AaIT—8 × 10^4 cpm.

first fraction represents intact AaIT. Radioactivity in this fraction corresponds to about 6.5% of the total radioactivity in the chromatogram. The third fraction behaves chromatographically as if it were iodide [12], but the results in Figure 1 indicate that radioactivity corresponds to a peptide which is a degradation product of the toxin similar to the second fraction.

Oral Treatment of Lepidopterous Larvae

For comparative purposes, a parallel experiment was performed with the lepidopterous larvae *Manduca sexta* (Sphingidae) and *Helioverpa* (=*Heliothis*) *zea* (Noctuidae). Although the tolerance of lepidopterous larvae to injected AaIT has already been described [12], we were interested in examining the permeability of their digestive system to ingested toxin. When hemolymph from [^{125}I]AaIT-fed *Manduca* larva chromatographed on paper, the radioactivity ran with intact [^{125}I]AaIT, coincident with radioactivity from *Sarcophaga* hemolymph (Fig. 1). On gel permeation chromatography, however, *Manduca* hemolymph contained only one radioactive peak, at an elution volume of 20 ml (the inclusion volume). The same results were obtained with hemolymph from *Helioverpa* (results not shown). Furthermore, when hemolymph was collected from *Manduca* or *Helioverpa* larvae that had been fed AaIT (10–20 μg/200 mg body weight), then injected into blowfly larvae, it failed to induce

contraction paralysis, a typical response of those larvae to AaIT [11,17]. This was in contrast to hemolymph collected from the above lepidopterous larvae injected with AaIT; this hemolymph did result in contraction paralysis [12; unreported results].

Crossing the Gut

The alimentary canal of *S. falculata* is a long, convoluted tube about three times the length of the body [18]. The gut is differentiated into three major regions: the foregut, the midgut, and the hindgut. The midgut, where the digestion and absorption of food occur, comprises about 90% of the length of the alimentary canal. Part of the tubular midgut shows a helix-like coiling in the abdominal cavity. In a previous study [3], the midgut of *Sarcophaga* was subdivided into several segments according to their permeability to the cardiotoxin. Segment 1, the cardiotoxin-permeable segment, included the frontal part of the midgut and the upper loop (from dorsal view) of the helical coil; segment 2, the nonpermeable segment, occupied the middle loop of the coil; and segments 3 and 4, the partially permeable segments, occupy the lower (ventral) and terminal loops of the helical coil [3].

In a previous study [3], it was demonstrated that free iodine (Na[^{125}I]) is removed from the fly's gut by the process of tissue fixation and preparation. Thus, the photographic grains presently revealed in the gut of *Sarcophaga* correspond to degradation peptides and some chromatographically intact toxin (Fig. 2). However, as shown in Figure 3, gut permeability was exclusively limited to the first cardiotoxin-permeable segment of the midgut, which revealed a cytology typical of that segment. The possibility that the nonpermeable segments of the gut were simply not accessible to the radioactive toxin was excluded by preliminary observations on the exposed intestine of flies (Materials and Methods), indicating the presence of dye-toxin solution throughout the entire length of the gut, and the obvious presence of the photographic grains in the lumen of the permeable as well as nonpermeable segments above and below the layers of the peritrophic membrane (Fig. 3–C,D).

DISCUSSION

Toxicity of AaIT to *Sarcophaga*

The data presented in Table 1 indicate that the crude venom and its derived AaIT were both orally toxic to adult flies, although the toxicity for both substances by injection was nearly three orders of magnitude stronger than the respective oral toxicities. The ratio of toxicity of the crude venom to toxicity of AaIT may indicate that the crude venom contains factors lethal to flies in addition to AaIT, and that AaIT is the major paralytic factor in the crude venom. Assuming that the oral toxicity of AaIT is a consequence of penetration across the gut epithelium, the ratio of oral to injection toxicities for AaIT suggests that 0.14% of the orally introduced toxin crossed the gut in a fully functional form (Table 1).

These observations raised questions concerning the degree and rate of penetration of the toxin through the gut, and those questions were addressed by employing radioiodinated toxin as a tracer.

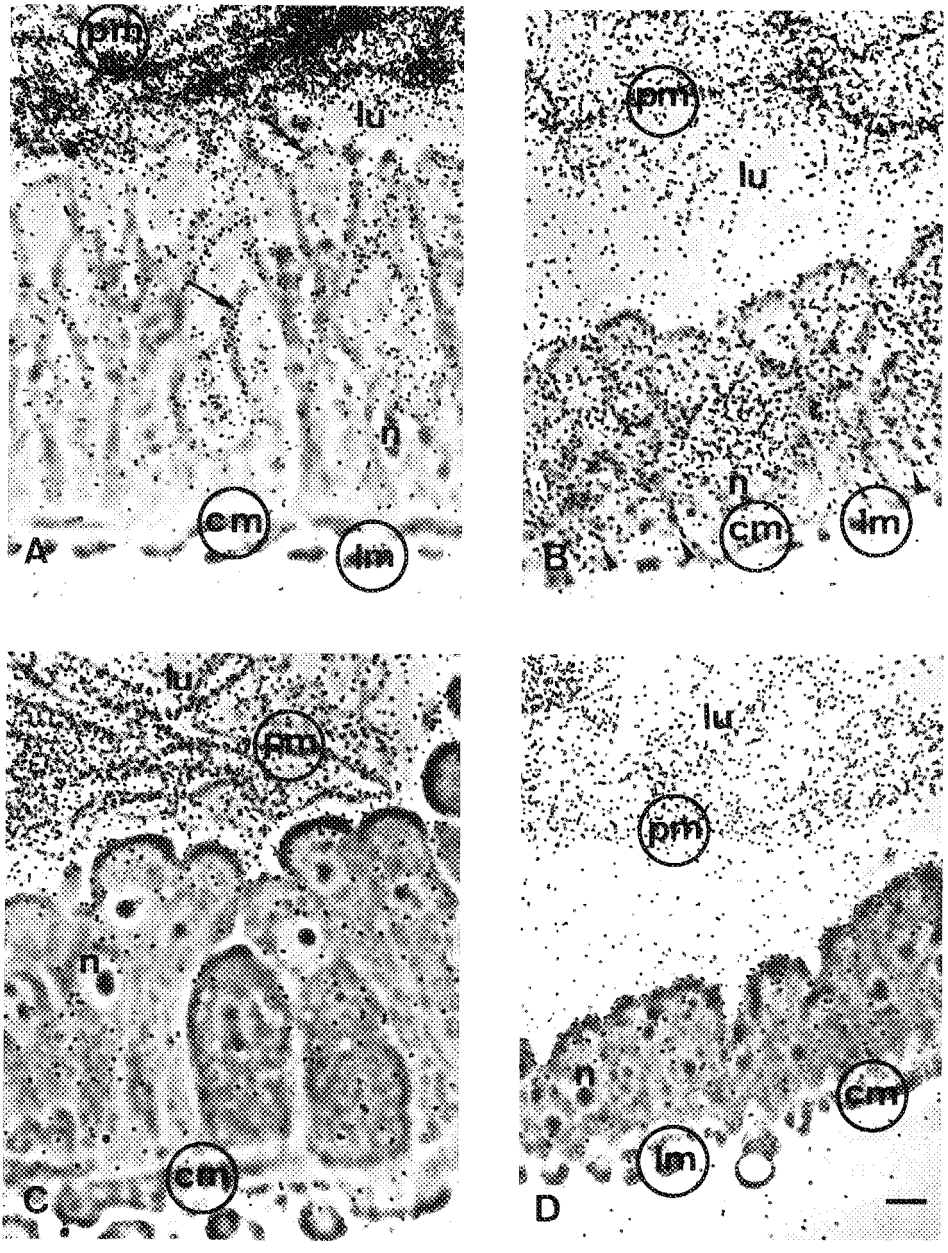

Fig. 3. Autoradiography of the midgut of *S. falculata* flies fed [^{125}I]AalT (3 μCi). Sagittal sections from different segments of the midgut were prepared. **A:** Segment 1, 7 min after application. This segment includes the frontal part of the midgut and the upper loop of the helical coil and is permeable to cardiotoxin [3]. Massive presence of [^{125}I]AalT in the lumen (lu) beyond the peritrophic membrane (pm) and the beginning of the entry into cells can be noticed. Photographic grains are located on the plain of the striated border and the apical region of the cells (arrows). cm, circular muscle; lm, longitudinal muscles; n, nucleus. **B:** Segment 1, 60 min after application. Photographic grains are present throughout the full length of the epithelial cells and appear in close vicinity to the circular muscles (cm, see arrowheads) enveloping the midgut. pm, peritrophic membrane; lu, lumen; lm, longitudinal muscles; n, nucleus. **C:** Segment 2, 60 min after application; same fly as in B. According

Gut Penetrability by AaIT

The results obtained with the radioiodinated toxin (Figs. 1, 2, Table 2) indicate that within 1–2 h after oral treatment about 5% of the ingested radioactivity from [^{125}I]AaIT was found in the hemolymph. This indicates that the toxin crosses the fly's gut in at least two different forms: first, in the form of small degradation peptides (Figs. 1, 2) that represent the vast majority (close to 95%) of the radioactivity present in the hemolymph; second, in the form of an intact, nondegraded substance, revealed by gel permeation chromatography (Fig. 2), corresponding to about 6–7% of the radioactivity in hemolymph and about 0.3% of the total orally applied [^{125}I]AaIT. As indicated by the above injection/oral application ratio (Table 1), it appears that the chromatographically intact form of the toxin possesses about 50% of the toxicity of the authentic AaIT. This suggests that the proportion of intact AaIT (0.3%) in the fly's hemolymph may be further subdivided into fully functional, partially inactivated, and fully inactivated forms. Such heterogeneity in the AaIT that penetrates the midgut is in contrast to the relative homogeneity revealed by cardiotoxin. The combination of toxicity [1] and binding assays [2] allowed the chemical and pharmacological identification and quantification of orally applied cardiotoxin in tissues of the *Sarcophaga* flies. The latter suggested gut penetrability of about 8% of the orally applied cardiotoxin in fully functional form within 3 h, which is about 50 times higher than the estimated penetrability of functional AaIT. The difference in gut penetrability of the two functional toxins may be attributed to resistance of the cardiotoxin to gut proteolytic enzymes. It is noteworthy that another low molecular weight (M_r 7,000) basic polypeptide derived from cobra venom, an a -neurotoxin which is nontoxic to insects [19], was shown to cross the fly's gut, but to a lesser degree and at a slower rate (2.2% within 48 h) [20].

Gut Morphology and Regional Permeability

Considering the fragmented vs. homogenous modes of permeability of AaIT and cardiotoxin, respectively, the morphological identity of their routes of penetration is surprising. The results presented in Figure 3 clearly indicate that [^{125}I]AaIT and its degradation products cross the *Sarcophaga* midgut exclusively through a cardiotoxin-permeable segment. The autoradiographical results indicate that both toxins were substantially absorbed by the same epithelial gut cells and revealed a frontal mode of entry through diffusion [3] (Fig. 3).

The anatomical and histological specificity of *Sarcophaga* gut permeability to the cardiotoxin has been interpreted in terms of a hypothetical specific composition and arrangement of phospholipids in the outer plasma mem-

to Fishman et al. [3], this segment corresponds to the centrally located loop of the helical coiling and is not permeable to cardiotoxin. As seen, it is impermeable to [^{125}I]AaIT. The single grains that can be seen in the cells correspond to the background radioactivity (data not shown). lu, lumen; pm, peritrophic membrane; n, nucleus; cm, circular muscle. **D:** Segment 4, 60 min after application; same fly as in B and C. This segment corresponds to the posterior part of the *Sarcophaga* midgut, which is partially permeable to cardiotoxin [3]. As shown this segment does not reveal any permeability to the [^{125}I]AaIT. lu, lumen; pm, peritrophic muscle; n, nucleus; cm, circular muscle; lm, longitudinal muscles. Bar: 10μm.

branes of the epithelial cells in the cardiotoxin-permeable segment of the midgut [3]. This hypothesis was supported by two additional pieces of information. First, histopathological changes induced by feeding large quantities of the cardiotoxin were limited to the cardiotoxin-permeable segment of the gut [3]. Second, another gut-penetrable protein of a higher molecular weight, horseradish peroxidase, Type II (M_r 40,000) (Sigma, St. Louis, MO), did not reveal specificity for any given region of the *Sarcophaga* midgut [21]. The present results concerning gut permeability to AaIT contradict the above hypothesis of cardiotoxin specificity, since AaIT is an entirely different polypeptide with regard to its chemistry and pharmacology (see Introduction), and AaIT fragments follow the same route of transport as intact AaIT and cardiotoxin polypeptides. The cardiotoxin-permeable segment of the midgut thus may be functionally specialized for the transport of low molecular weight peptides. This hypothesis demands further exploration. Morphologically distinct regions in the insect midgut have been found in the tsetse flies [22], culicid mosquitos [23], *Phlebotomus* flies [24], and the phasmid *Carausius* [25]. In view of the present results, it appears that the distinctive morphology noted above may possess a functional significance deserving of experimental clarification.

Toxicological Aspects

Despite the relative vulnerability of AaIT to proteolytic digestion when compared to cardiotoxin, its oral toxicity to *Sarcophaga* is about 2.5-fold higher than that of cardiotoxin (Table 1) [2], since toxicity by injection of AaIT is about 150 times higher than that of the cardiotoxin [1,2]. Furthermore, it has recently been shown that AaIT is toxic to houseflies through topical application (1 μg/100 mg body weight) which is 1.5 times more active than propoxur and 10 times more active than DDT or malathion [6]. The ratio of topical to injection toxicities of AaIT in houseflies (500) [6] resembles the ratio of oral to injection toxicities in *Sarcophaga* flies (≈ 700) (Table 1). Results with lepidopterous larvae (Figs. 1, 2) indicate that AaIT is fully degraded by strong proteolytic activity in their digestive systems, but these results do not exclude possible permeability to other polypeptides. It appears, therefore, that structural modifications (through either synthetic or genetic approaches) resulting in a metabolic stabilization of AaIT may highly increase its oral and topical toxicities.

The results concerning oral and topical toxicities of AaIT, even when limited to flies, in essence reveal the potential feasibility of employing neurotoxic polypeptides for insect control purposes [26]. The insect selectivity and the neurotoxic action of AaIT and other related polypeptides [27,28] should encourage efforts toward biochemical stabilization and development of delivery techniques.

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